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# Hybrid penicillin acylases with improved properties for synthesis of $\beta$ -lactam antibiotics

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## Abstract

Penicillin acylase (PA) from *Escherichia coli* can catalyze the acylation of 6-aminopenicillanic acid (6-APA), a conversion that is applicable in the biocatalytic preparation of semi-synthetic  $\beta$ -lactam antibiotics such as ampicillin. The efficiency of this kinetically controlled conversion, in which an amide or ester acts as the acyl donor, is dependent on the kinetic properties of the enzyme. To further improve the synthetic properties of PAs, family gene shuffling was performed with the PA-encoding genes of the PAs from *E. coli*, *Kluyvera cryocrescens* and *Providencia rettgeri*. Of these three PAs, the *E. coli* enzyme possessed the best properties for the synthesis of ampicillin. Shuffled recombinant libraries were pre-screened for activity by growth selection, followed by testing the catalytic performance in ampicillin synthesis using HPLC. Three clones with improved synthetic properties were selected and sequence analysis showed that the shuffled genes were hybrids of the PA-encoding genes from *E. coli* and *K. cryocrescens*, with additional point mutations. The hybrid enzymes displayed a 40–90% increase in the relative rate of acyl transfer to the  $\beta$ -lactam nucleus during ampicillin synthesis. This increase was not accompanied by a reduction of synthetic activity that has previously been reported for mutants of *E. coli* PA constructed by site-directed mutagenesis. Similar improvements in acyl transfer were obtained for the synthesis of amoxicillin, cephalexin and cefadroxil, making the new hybrid enzymes interesting candidates for the biocatalytic synthesis of several  $\beta$ -lactam antibiotics.  
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**Keywords:** Penicillin acylase; Ampicillin; Cephalexin; Synthesis; Biocatalysis; Gene shuffling; Directed evolution

## 1. Introduction

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) is a serine hydrolase that is used for the biocatalytic production of 6-aminopenicillanic acid (6-APA) by hydrolysis of penicillin G, which is obtained by fermentation. Penicillin acylases (PA) are present in various bacteria, archaea and fungi [1–3], and they are likely to be involved in the metabolism of aromatic carbon compounds [4,5]. The *pac* gene encodes a preprotein consisting of a signal peptide, an  $\alpha$ -chain, a spacer peptide and a  $\beta$ -chain and maturation of this PA preprotein yields a heterodimer consisting of the  $\alpha$ - and  $\beta$ -chain of 23 and 63 kDa, respectively. In 2000, the crystal structure of the precursor protein was solved and it provided evidence for an autocatalytic processing mechanism of the preprotein [6]. Earlier X-ray analysis had already

shown that the hydroxyl group of the N-terminal serine of the  $\beta$ -subunit becomes acylated during the first half reaction, and deacylated when the acyl enzyme is cleaved in the second half reaction [7]. The free  $\text{NH}_2$  of this serine is proposed to act as a base that facilitates proton abstraction.

The penicillin nucleus 6-APA is a key intermediate for the preparation of several semi-synthetic  $\beta$ -lactam antibiotics. Chemical coupling of an appropriate acyl group to 6-APA is accompanied by the use of hazardous and polluting chemicals, needs to be done at low temperature to prevent formation of side-products, and requires a large amount of energy [8–10]. The use of biocatalysis would make this conversion more environmentally benign. Penicillin acylase can also be applied for the coupling of an acyl group to 6-APA. Unfortunately, the synthetic properties of the well-studied PA from *Escherichia coli* ATCC 11105 are only moderate, which causes incomplete conversion of the substrates to products. Since the enzymatic coupling is a kinetically controlled process, the yield of the reaction is dependent on the kinetic properties of the enzyme, and alternative enzymes may be more suitable. Penicillin acylases have also

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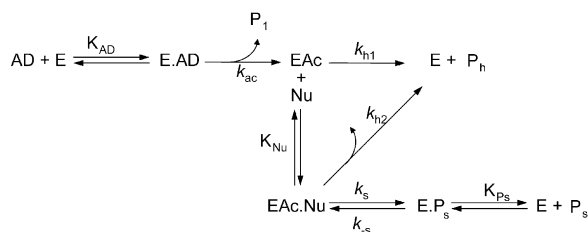


Fig. 1. Kinetic scheme describing the synthesis and hydrolysis reactions catalysed by PA. Abbreviations: E is the free enzyme, E.AD is the non-covalent enzyme-acyl donor complex,  $P_1$  is the product released during acylation of the enzyme, EAc is the acyl-enzyme intermediate, A the hydrolysis product, EAc.Nu is the non-covalent acyl-enzyme-nucleophile complex, E.P<sub>s</sub> is the non-covalent enzyme-product complex and P<sub>s</sub> is the synthesis product (e.g., antibiotic).  $K_{Nu}$ ,  $K_{AD}$  and  $K_{Ps}$  are the binding constants of substrates and products indicated, and  $k_{ac}$ ,  $k_{h1}$ ,  $k_s$ ,  $k_{h2}$  and  $k_{h2}$  are the rate constants of the reaction steps.

been found in *Kluyvera cryocrescens* and *Providencia rettgeri*, but the synthetic properties of these enzymes are not well documented.

Youshko et al. [11] proposed that a kinetically controlled synthesis catalyzed by PA can be represented by the scheme shown in Fig. 1. Essentially the same scheme was used to describe

the synthesis and hydrolysis of peptides by chymotrypsin [12], papain [13], and carboxypeptidase Y [14]. An activated acyl donor (AD), usually an amide or an ester of a phenylacetic acid derivative, is attacked by Oδ of βSer1 of the enzyme to form an acyl-enzyme intermediate (EAc) (Fig. 2). This covalent intermediate can either be hydrolyzed by water, yielding the free acid (P<sub>h</sub>) of the acyl donor, or aminolyzed by a β-lactam nucleus (Nu), yielding the desired product (P<sub>s</sub>). The formed antibiotic can also be hydrolyzed by the enzyme, liberating the β-lactam nucleus (Nu) and the free acid (P<sub>h</sub>). Due to this competitive product hydrolysis, the concentration of antibiotic will reach a maximum  $[P_s]_{max}$ , after which it will decrease again (Fig. 3). Since no antibiotic is present at the beginning of the reaction, product hydrolysis can be neglected in the initial phase of the conversion. Hence, the rate of formation of P<sub>s</sub> ( $v_{P_s}$ ) divided by the rate of formation of P<sub>h</sub> ( $v_{P_h}$ ) represents the preference of the acyl-enzyme for the β-lactam nucleus over water in deacylation and is given by Youshko et al. [15]:

$$\left( \frac{v_{P_s}}{v_{P_h}} \right)_{ini} = \frac{\beta_0[Nu]}{1 + \beta_0\gamma[Nu]} \quad (1)$$

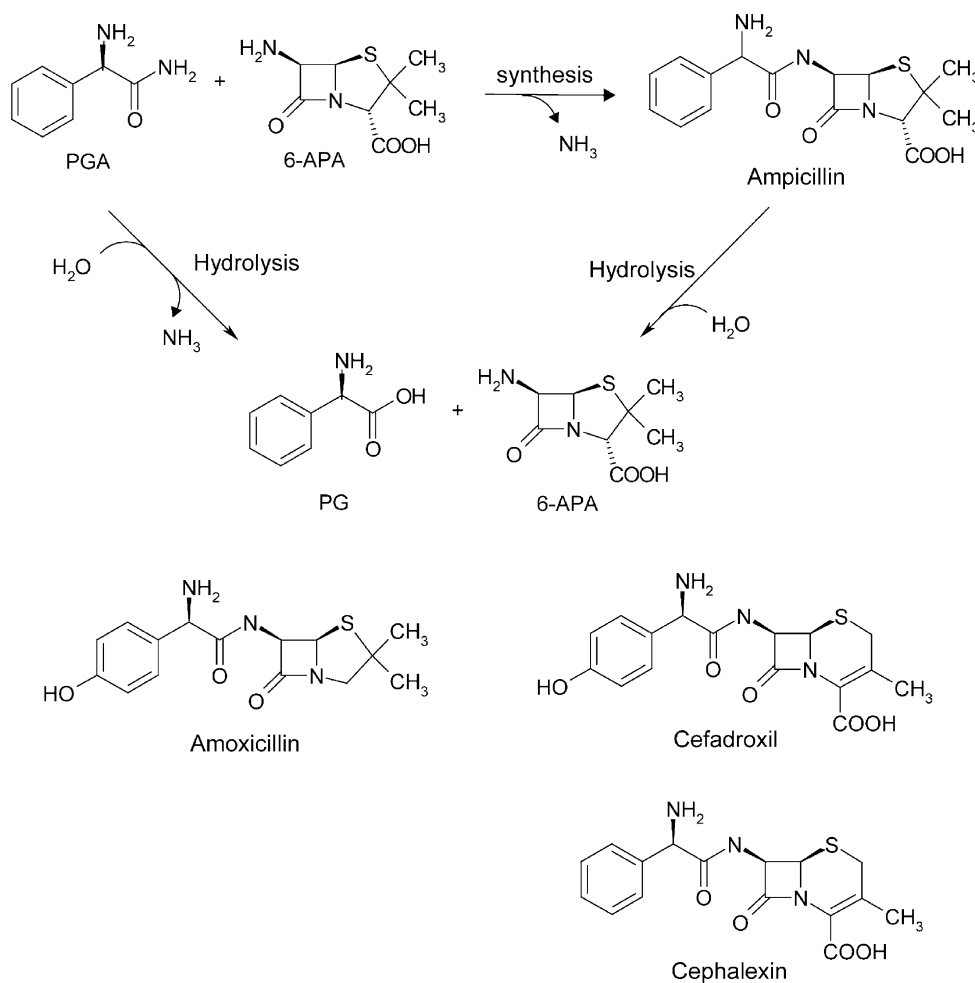


Fig. 2. Kinetically controlled synthesis of ampicillin. Penicillin acylase can couple the activated side chain (PGA) to the β-lactam nucleus (6-APA). Both the activated side chain and the formed product can be hydrolyzed to phenylglycine (PG). Structures of the other semi-synthetic β-lactam antibiotics that were synthesized by the hybrids are given as well.

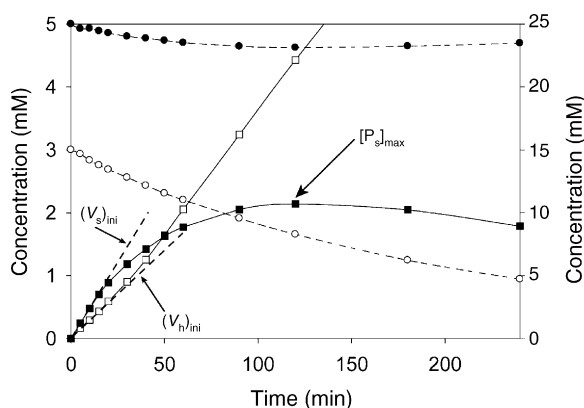


Fig. 3. Synthesis of ampicillin (■) and the formation of PG (□) catalyzed by *E. coli* PA from 15 mM D-PGA (○) and 25 mM 6-APA (●). The left y-axis shows the concentrations of the products ampicillin and D-PG, whereas the right y-axis shows the concentrations of the substrates D-PGA and 6-APA. The dashed lines represent the initial rate of formation of the products and the synthetic parameters  $(P_s)_{\max}$ ,  $(v_s)_{\text{ini}}$  and  $(v_h)_{\text{ini}}$  are depicted.

According to Eq. (1), the plot of  $(v_p/v_h)_{\text{ini}}$  against  $[\text{Nu}]$  increases hyperbolically with increasing nucleophile concentrations. At low levels of the  $\beta$ -lactam nucleus (Nu), most of the acyl enzyme is in the unbound form. Hence, the covalent intermediate is mainly hydrolyzed by water. At increasing concentrations, the  $(v_p/v_h)_{\text{ini}}$  reaches a maximum when the acyl enzyme is saturated with the nucleophile (Nu). This maximum is given by  $1/\gamma$ , whereas  $\beta_0$  corresponds to the initial slope [15].

The third parameter necessary to describe the behaviour of PA in a kinetically controlled synthesis is the factor  $\alpha$ , which is given by:

$$\alpha = \frac{(k_{\text{cat}}/k_{\text{m}})_{\text{P}_s}}{(k_{\text{cat}}/k_{\text{m}})_{\text{AD}}} \quad (2)$$

A low  $\alpha$ -value indicates a relatively low tendency of product hydrolysis, which leads to an increase of the maximum amount of product that is accumulated during the conversion.

With these three parameters the maximum amount of product that accumulates during a kinetically controlled synthesis can be predicted according to Eq. (3) [15].

$$\frac{d[\text{P}_s]}{d[\text{P}_h]} = \frac{\beta_0[\text{Nu}][\text{AD}] - \alpha[\text{P}_s](1 + \beta_0\gamma[\text{Nu}])}{(1 + \beta_0\gamma[\text{Nu}])([\text{AD}] + \alpha[\text{P}_s])} \quad (3)$$

with  $[\text{AD}]_0 = [\text{AD}] + [\text{P}_s] + [\text{P}_h]$  and  $[\text{Nu}]_0 = [\text{Nu}] + [\text{P}_s]$ . The formulas show that  $\beta_0$  and  $1/\gamma$  should have high values and  $\alpha$  should have a low value for optimal synthetic performance of PA.

In recent years, a number of mutants with altered catalytic properties and in some cases an improved ratio between the rates of synthesis and hydrolysis have been constructed by site-directed mutagenesis [16,17], but an important drawback of these variants of *E. coli* PA is that they have a greatly reduced synthetic activity ( $v_p$ ). In this paper we present hybrid PAs that were constructed with the use of gene shuffling. Three PA-encoding genes from *E. coli*, *K. cryocrescens* and *P. rettgeri*

were used as starting sequences to construct a pool of chimeric genes. From this library, the transformants with the best catalytic properties for the synthesis of ampicillin were selected and their catalytic performance was investigated and compared with that of the parent enzymes.

## 2. Materials and methods

### 2.1. Strains and plasmids

The gene encoding PA of *E. coli* holds three mutations compared to the gene sequence in the GenBank (accession number 42247): T815C (encoding V272A), G1309C and G1311A (encoding V437L). The pEC vector carries the PA-encoding gene from *E. coli*, as well as a chloramphenicol acetyltransferase (*cat*) gene as resistance marker and a *tacI* promoter that can be induced by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) [18,19]. The DNA that encodes PA from *K. cryocrescens* ATCC 21285 was amplified from genomic DNA, using the primers KCFw (5'-CTGCAAGAGGATCATATGAAAAATAGA-3', *NdeI*-site in italics) and KCRv (5'-GGGGAGGCCGAAAGCTTAGCGCTGTAC-3', *HindIII*-site in italics). After amplification and restriction, the gene was cloned in the pEC vector that was previously cut with the same restriction endonucleases, yielding plasmid pKC. The DNA encoding the PA from *P. rettgeri* ATCC 9250 was amplified from genomic DNA. The primers used for the amplification were PRFw (5'-CATGGGACTCAACATATGAAAAACACCTC-3', *NdeI*-site in italics) and PRRv (5'-TTAGCTTAACCCGGGTATTATCTCTCAAT-3', *SmaI* site in italics). Subsequently, the gene was cloned in pEC, yielding the plasmid pPR. *E. coli* HB101 was used as host for all plasmids.

### 2.2. Gene shuffling

Amplification of the ancestral genes was done using 1 U *Taq* polymerase (Roche Diagnostics GmbH, Mannheim, Germany). *Taq* DNA polymerase was used in order to introduce mutations at random positions. The forward primer used for amplifying all genes was the 27-mer 5'-GAAACAGGATCCAAGGAAAAACATATG-3', which anneals partially in front of and partially on the PA genes. It contains a *NdeI*-restriction site (italics). As reverse primer three different oligonucleotides were used: for pEC the 33-mer 5'-CAGGTCGACCCCGGTTATCTCTGAACGTGCAA-3', for pKC the 33-mer 5'-CAGGTCGACCCCGGTTAGCGCTGTACCTGCAG-3', and for pPR the 33-mer 5'-CAGGTCGACCCCGGTTATCTCTCAATTATTAG-3'. These primers contain both a *SalI* (italics) and *SmaI* restriction site (italics and underlined). The PCR reaction was performed using a Progene Thermo cycler (Techne, Cambridge, UK) employing the following program: 10 min at 94 °C, 25 cycles of 1.0 min at 94 °C, 1.5 min at 55 °C and 2.5 min at 72 °C, followed by 5 min at 72 °C.

For DNase digestion, a mixture of 1  $\mu\text{g}$  of each parent DNA was incubated with 0.15 U DNase (Roche, Basel, Switzerland) at 30 °C for 7.5 min. After the digestion, the DNase was inactivated by heating the mixture at 95 °C for 15 min and fragments of 50–400 base pair (bp) were purified from a 2% agarose gel (Gibco, Inchinnan, Paisley, UK), using a PCR purification kit (Qiagen GmbH, Hilden, Germany).

For the reassembly reaction, about 1  $\mu\text{g}$  of purified digested DNA was used in a PCR reaction without primers. The reassembly was performed using a Progene Thermo cycler in a total volume of 30  $\mu\text{l}$  employing the following program: 1.0 min at 94 °C, 80 cycles of 1.0 min at 94 °C, 1.0 min at 35 °C and 2.5 min at 72 °C, followed by 5 min at 72 °C.

To introduce restriction sites for cloning of the full-length products, 1  $\mu\text{l}$  of the reassembly mix was used in a subsequent PCR reaction with the forward primer 5'-GAAACAGGATCCAAGGAAAAACATATG-3' (*NdeI*-site shown in italics), and the reverse primer 5'-TTCGACCTCTAGACCAAGCTTCTGACGTCGACCCCGGG-3' (*SmaI*-site italics, a *SalI*-site in italics and underlined, a *HindIII*-site in bold, *XbaI*-site bold and underlined). The polymerase used was *Pwo* (Roche) and the employed program was the same as for the initial amplification of the parent genes. After digesting the PCR product with *NdeI* and *XbaI*, it was ligated into pEC that was digested with the same enzymes.

### 2.3. Selection of active PAs

Competent *E. coli* HB101 cells were transformed using standard protocols and plated on minimal medium supplemented with 20 mM glucose as carbon and energy source, 1 mM  $\text{NH}_4\text{Cl}$  as nitrogen source, 10 mg/l proline, 20 mg/l *N*-phenylacetyl-L-leucine and 68 mg/l chloramphenicol. Colonies that had appeared after 3 days incubation at 30 °C were transferred to 96-well microtiter plates containing 250  $\mu\text{l}$  LB medium and 68 mg/l chloramphenicol per well and allowed to grow at 37 °C for 24 h at 200 rpm. After addition of glycerol to a final concentration of 10%, the plates were stored at –80 °C. Each microtiter plate contained 84 transformants, the three parents in duplicate, duplicate negative controls, and four wells containing only LB medium.

### 2.4. Screening for improved mutants

From the stored microtiter plates, 10  $\mu\text{l}$  cell suspension was transferred to a second microtiter plate containing 240  $\mu\text{l}$  LB medium supplemented with 68 mg/l chloramphenicol and 0.1 mM IPTG and the cells were allowed to grow for 36 h at 16 °C and 200 rpm. Subsequently, 100  $\mu\text{l}$  culture was mixed with 100  $\mu\text{l}$  of HPLC reaction mixture, consisting of 200 mM D-phenylglycine amide (D-PGA) and 75 mM 6-aminopenicillanic acid in 50 mM phosphate buffer, pH 7.0, and incubated at 30 °C. After 2, 4 and 8 h, samples were taken and quenched using a Plato 3001 automated pipetting station (Rosys AG, Switzerland) that also functioned as a 96-well microtiter plate format autosampler for HPLC injection. The samples were analyzed by HPLC on an Alltima C18 rocket column in connection with a Jasco PU-1586 pump and a Jasco UV-1586 detector set at 214 nm. All compounds were eluted isocratically using a solution containing 340 mg/l SDS, 5 mM phosphate, 30% acetonitrile, and adjusted to pH 3.0 with phosphoric acid. Concentrations of PG and ampicillin were determined and the ratio of the ampicillin concentration over the PG concentration ( $[P_s]/[P_h]$ ) was calculated.

For preparation of periplasmic extracts containing PA, cells were grown at 17 °C in 50 ml LB medium supplemented with 0.1 mM IPTG. The cells were harvested by centrifugation at 6000 rpm for 10 min and the pellet was suspended in 25 ml of ice-cold osmotic shock buffer A (20% sucrose and 10 mM EDTA in 100 mM Tris–HCl, pH 8.0) and centrifuged at  $3600 \times g$  for 10 min. The pellet was resuspended in 1 ml of ice-cold osmotic shock solution B (1 mM EDTA) and centrifuged at  $3600 \times g$  for 10 min. Potassium phosphate buffer (1 M, pH 7.0) was added to the supernatant, which is called a periplasmic extract, to a final concentration of 50 mM. Enzyme concentrations were determined by titration with phenylmethylsulfonyl fluoride (PMSF) [20]. For purification of PA, 1 L cultures were grown in LB medium supplemented with 0.1 mM IPTG at 17 °C. The enzymes were purified as described [17].

The synthesis experiments were carried out with 100 nM enzyme at 30 °C in 50 mM potassium phosphate buffer, pH 7.0, and the reaction was started by addition of 6-APA and D-PGA to a final concentration of 25 and 15 mM, respectively. Samples were taken and analyzed by isocratic HPLC, using a Chrompack C18 column in connection with a Jasco PU-980 pump and a Jasco UV-1575 detector set at 214 nm.

### 2.5. Kinetic analysis

All enzymatic reactions were carried out in a 50 mM phosphate buffer, pH 7.0. Synthesis was started after mixing 100 nM enzyme with 25 mM nucleophile, which was either 6-APA or 7-aminodesacetoxycephalosporanic acid (7-ADCA) and 15 mM acyl donor, either D-phenylglycine methylester (D-PGM), D-phenylglycine amide (D-PGA) or D-*p*-hydroxyphenylglycine amide (D-HPGA). Samples were taken and analyzed by isocratic HPLC. The determination of  $\beta_0$  and  $\gamma$  was done in a series of conversions with 15 mM D-PGA and varying 6-APA concentrations.

The hydrolysis of 2-nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB) and 2-nitro-5-[(phenylglycyl)amino]-benzoic acid (NIPGB) was monitored using a Perkin-Elmer Lambda Bio 40 spectrometer. The hydrolysis of NIPAB and NIPGB by PA liberates 5-amino-2-nitrobenzoic acid, which can be measured at 405 nm [21]. The extinction coefficient of 5-amino-2-nitrobenzoic acid is  $9.09 \text{ mM}^{-1} \text{ cm}^{-1}$  at pH 7.0. The data was fitted using the program Sigmaplot

(SPSS Inc.). Steady state kinetic parameters of D-PGA and ampicillin were determined using HPLC. Values for  $\alpha$  were obtained from the ratios between  $k_{\text{cat}}/K_m$  for acyl donor and product (Eq. (2)). The inhibition constant of phenylacetic acid (PAA) was determined as described by Alkema et al. [16].

Simulations of maximal levels of product accumulation as a function of kinetic parameters  $\alpha$ ,  $\beta$ , and  $\gamma$  were performed using the program Mathematica (Wolfram Research, Champaign, IL).

### 2.6. Chemicals

NIPAB and NIPGB were purchased from Syncom (Groningen, The Netherlands). PMSF was from Serva (Heidelberg, Germany). 6-APA, 7-ADCA, D-PGA, D-PGM and D-HPGA were a gift from DSM-Gist (Delft, The Netherlands). IPTG was purchased from Roche.

## 3. Results

### 3.1. Gene shuffling of penicillin acylases

Penicillin acylases can be used to transfer an acyl group of a carboxylic acid ester or amide to 6-aminopenicillanic acid or other  $\beta$ -lactam nuclei, but the kinetic properties of the intensively studied PA from *E. coli* make the enzyme only moderately suitable for synthetic conversions. This is mainly due to the competition between water and 6-APA for the acyl-enzyme intermediate and product hydrolysis. To find better variants than the wild-type enzyme, the three PA encoding genes of *E. coli*, *K. cryocrescens* and *P. rettgeri* were subjected to family gene shuffling [22,23]. The enzymes encoded by these genes individually do not have better synthetic properties than the *E. coli* PA (see below). The genes share 77% (*E. coli* and *K. cryocrescens*), 61% (*K. cryocrescens* and *P. rettgeri*) and 60% (*E. coli* and *P. rettgeri*) sequence identity at the DNA level. To eliminate unnecessary screening of inactive transformants a growth selection was applied prior to the more time consuming HPLC measurements of the recombinants' synthetic properties. For this, the leucine auxotrophic host *E. coli* HB101 was used in combination with minimal medium agarose plates supplemented with 20 mg/l *N*-phenylacetyl-L-leucine as the leucine source. The parental PAs can liberate sufficient L-leucine to complement the auxotrophic marker [24,25]. To check the percentage of active recombinants, equal amounts of transformed *E. coli* HB101 cells were plated on LB medium and on minimal medium supplemented with *N*-phenylacetyl-L-leucine. Only 3% of the colonies that appeared on LB medium also grew on minimal medium, thus only a small fraction of the shuffled recombinants produced active PA.

Active transformants were grown in microtiter plates and cell suspensions were incubated with D-PGA and 6-APA to screen for ampicillin production by HPLC. Using the protocol described in Section 2, one microtiter plate was analyzed in 20 h. From the 700 active transformants that were tested, 94% showed detectable (more than 2% compared to *E. coli* PA) ampicillin synthesis within 8 h. This indicates that the majority of the transformants that were able to liberate leucine from (*N*)-phenylacetyl-leucine could also synthesize ampicillin from D-PGA and 6-APA, demonstrating the validity of the growth selection that was used for pre-screening. The  $[P_s]/[P_h]$ -ratio was calculated at three time points and compared to that of the wild-



Table 1  
Synthetic properties of parent and hybrid penicillin acylases

Enzyme <sup>d</sup>	Ampicillin <sup>a</sup>				Ampicillin <sup>b</sup>				Amoxicillin <sup>c</sup>				Cephalexin <sup>a</sup>				Cefadroxil <sup>c</sup>			
	$(v_{P_s}/v_{P_h})_{ini}$	$[P_s]_{max}$ (mM)	$v_{P_s}$ (%)	$(v_{P_s}/v_{P_h})_{ini}$	$[P_s]_{max}$ (mM)	$v_{P_s}$ (%)	$(v_{P_s}/v_{P_h})_{ini}$	$[P_s]_{max}$ (mM)	$v_{P_s}$ (%)	$(v_{P_s}/v_{P_h})_{ini}$	$[P_s]_{max}$ (mM)	$v_{P_s}$ (%)	$(v_{P_s}/v_{P_h})_{ini}$	$[P_s]_{max}$ (mM)	$v_{P_s}$ (%)	$(v_{P_s}/v_{P_h})_{ini}$	$[P_s]_{max}$ (mM)	$v_{P_s}$ (%)		
E	1.4	2.2	100	0.7	2.2	100	1.4	1.9	100	16.8	3.5	100	6.0	3.1	100					
K	1.3	2.1	110	0.6	1.6	59	1.8	1.9	78	21.8	3.4	85	6.7	3.0	76					
P	0.6	1.9	59	0.4	1.3	40	0.9	2.1	153	3.1	2.7	62	3.0	3.2	183					
6G8	2.1	2.6	149	0.9	2.5	121	2.0	2.4	139	33.9	4.2	118	8.3	3.7	103					
73C4	2.6	2.4	95	0.9	2.7	75	1.8	2.2	108	34.6	3.8	66	10.1	3.3	85					
6B11	2.0	2.5	115	0.8	2.4	85	2.1	2.3	83	19.7	3.6	77	7.8	3.4	62					

<sup>a</sup> 15 mM D-PGA as the acyl donor.

<sup>b</sup> 15 mM D-PGM as the acyl donor.

<sup>c</sup> 15 mM D-HPGA as the acyl donor.

<sup>d</sup> Enzymes: E, *E. coli*; K, *K. cryocrescens*; P, *P. rettgeri* penicillin acylase.

type enzymes. An increased  $[P_s]/[P_h]$ -ratio was observed to be correlated with an improved initial synthetic ratio  $((v_{P_s}/v_{P_h})_{ini})$  and was therefore used as a criterion for improved synthesis. Out of the 700 clones, 81 transformants (12%) synthesized ampicillin to a level of at least 50% of what was found with wild-type *E. coli* enzyme, while 19 of these 81 transformants also showed an improved  $[P_s]/[P_h]$ -ratio.

The 19 best clones were tested for antibiotic synthesis with periplasmic extracts, which were obtained by subjecting the cell suspensions to an osmotic shock. After the determination of the PA concentration by PMSF titration, progress curves of the formation of both ampicillin (synthesis product) and PG (hydrolysis product) were determined. From these progress curves, we derived the  $[P_s]_{max}$ , the initial  $v_{P_s}/v_{P_h}$ -value and the synthetic activity (Table 1), represented by the initial rate of ampicillin formation. All 19 selected transformants displayed better synthetic properties than PA of *P. rettgeri*. Compared to PA of *E. coli*, eight transformants showed an improved  $[P_s]_{max}$  or an improved  $(v_{P_s}/v_{P_h})_{ini}$ -value, and four transformants were improved in  $[P_s]_{max}$  as well as in  $(v_{P_s}/v_{P_h})_{ini}$ . Four transformants displayed a higher synthetic activity ( $v_{P_s}$ ) for ampicillin than the most active ancestral enzyme in ampicillin synthesis, that from *K. cryocrescens*. The three best transformants were named 73C4, 6B11 and 6G8. They were confirmed to be hybrids by sequencing (see below) and studied in more detail by comparison of the properties of the purified enzymes to the parent wild-type enzymes.

### 3.2. Ampicillin synthetic properties and kinetics

Of the wild-type enzymes, the penicillin acylases from *E. coli* and *K. cryocrescens* appeared to have a slightly higher initial rate of ampicillin formation ( $v_{P_s}$ ) and a lower initial  $v_{P_s}/v_{P_h}$  than the enzymes from *P. rettgeri*, while the three enzymes were similar with respect to the  $[P_s]_{max}$  (Table 1). The three selected hybrids showed a significantly improved  $(v_{P_s}/v_{P_h})_{ini}$  as compared to PA from *E. coli*. Hybrid 73C4 even displayed an 86% increase of the initial  $v_{P_s}/v_{P_h}$ . Furthermore, the maximum product yield ( $[P_s]_{max}$ ) of the hybrids was elevated up to 19% for hybrid 6G8. Hybrids 6G8 and 6B11 showed an increased synthetic rate ( $v_{P_s}$ ), whereas hybrid 73C4 showed only a slight decrease in synthetic activity. Thus, two out of the three mutants combine to form an improved  $[P_s]_{max}$  and  $(v_{P_s}/v_{P_h})_{ini}$  with a remarkable increase of the synthetic rate in ampicillin production.

The three parameters  $\alpha$ ,  $\beta_0$  and  $\gamma$ , which describe the synthetic behaviour of penicillin acylases in the kinetically controlled synthesis at various nucleophile concentrations, were determined for the wild-type enzymes and the hybrids (Table 2). Values for  $\alpha$  were obtained from kinetic parameters ( $k_{cat}/K_m$ ) for acyl donor and product, and values for  $\beta$  and  $\gamma$  were calculated from a plot of  $(v_{P_s}/v_{P_h})_{ini}$  against ampicillin concentration. As can be seen from Fig. 4, the  $(v_{P_s}/v_{P_h})_{ini}$  values of *E. coli* and *K. cryocrescens* PA are similar at lower nucleophile concentrations, whereas at higher concentrations the *E. coli* enzyme showed a slightly higher  $(v_{P_s}/v_{P_h})_{ini}$ -value. Consequently, the  $1/\gamma$ -value of *E. coli* PA is higher. The three hybrids, however, showed even better values for  $\gamma$ . Similarly, the values for  $\beta_0$  were better for

Table 2

Complex kinetic constants of the parent and hybrid enzymes for ampicillin synthesis

Penicillin acylase	$\alpha$	$\beta_0$ (mM <sup>-1</sup> )	$1/\gamma$
<i>E. coli</i>	7.8	0.078	7.2
<i>K. cryocrescens</i>	12.7	0.098	5.3
<i>P. rettgeri</i>	5.8	0.032	3.3
6G8	11.1	0.130	8.1
73C4	11.6	0.120	8.4
6B11	6.4	0.115	7.9

The factor  $\alpha$  is defined as the ratio of the specificity constants for ampicillin and D-PGA (See Table 3).

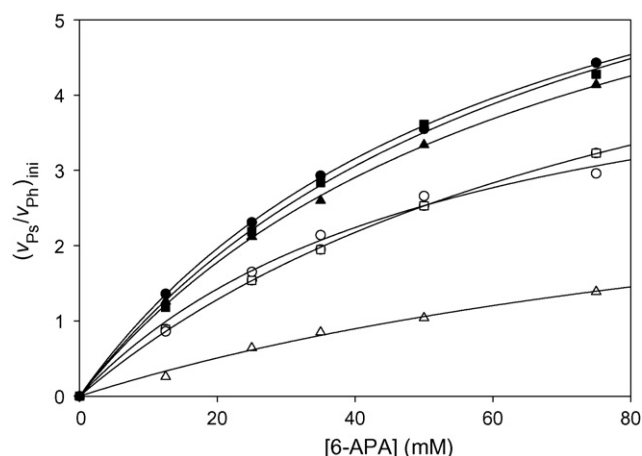


Fig. 4. Nucleophile reactivity of 6-APA in the synthesis of ampicillin by the ancestral and hybrid PAs. The initial rate of ampicillin formation and phenylglycine formation was measured in reactions containing 15 mM D-PGA and varying concentrations of 6-APA. Penicillin acylases: *E. coli* PA ( $\square$ ), *K. cryocrescens* PA ( $\circ$ ) and *P. rettgeri* PA ( $\triangle$ ); hybrid 6B11 ( $\blacktriangle$ ), hybrid 6G8 ( $\blacksquare$ ) and hybrid 73C4 ( $\bullet$ ).

the three hybrids than for the best wild-type enzyme, which was *K. cryocrescens* PA.

A low  $\alpha$ -value is one of the factors that leads to a high level of product accumulation [15]. For PA from *E. coli*  $\alpha$  was 7.8, implying that this enzyme has a higher specificity constant for the product than for the acyl donor, which causes significant product hydrolysis when its concentration increases and the acyl donor becomes depleted. Of the other enzymes, the PAs from *P. rettgeri* and hybrid 6B11 showed lower values for  $\alpha$  as compared to the *E. coli* enzyme, which was mainly due to a decrease in the specificity constant for ampicillin (Table 3). Mutant 73C4

displayed a 50% higher  $\alpha$  and this was caused by an increase in the  $K_m$  for D-PGA.

With these three parameters it is possible to predict the maximum ampicillin yield that can be reached using various substrate concentrations (Eq. (3)). The results of these simulations show that all hybrids showed improved synthetic properties at low substrate concentrations, as was established experimentally (Fig. 5). At high substrate concentrations, however, hybrids 6G8 and 73C4 did not perform better than PA from *E. coli*, indicating that  $\alpha$ , which was increased for these hybrids, plays a more important role at higher substrate concentrations. Hybrid 6B11 is significantly improved with respect to  $[P_s]_{\max}$ . For example, PA of *E. coli* is predicted to give an ampicillin yield of 50 mM from 200 mM 6-APA and 200 mM D-PGA. Hybrid 6B11 shows this yield already with only 160 mM acyl acceptor and 200 mM D-PGA.

Ampicillin can also be synthesized using D-phenylglycine methylester (D-PGM) as the acyl donor and 6-APA as the nucleophile (Table 1). In this reaction PA from *E. coli* again performed the best of the wild-type enzymes. The two other enzymes showed a lower  $(v_{P_s}/v_{P_h})_{\text{ini}}$ , a lower level of ampicillin accumulation ( $[P_s]_{\max}$ ) and a lower synthetic activity than the *E. coli* enzyme and the level of product accumulation was lower than when D-PGA was used as the acyl donor (Table 1). The three selected hybrids showed better synthetic properties than the wild-type enzymes, even though the screening of the library was only based on their ability to synthesize ampicillin from D-PGA and 6-APA. The synthetic rate of hybrid 6G8 appeared to be 20% higher than that of the *E. coli* wild-type enzyme, and both  $(v_{P_s}/v_{P_h})_{\text{ini}}$  and  $[P_s]_{\max}$  were increased for all three hybrids.

### 3.3. Synthesis of other antibiotics

To examine if the mutants were also able to synthesize other  $\beta$ -lactam antibiotics than ampicillin, the synthesis of amoxicillin, cephalexin and cefadroxil was tested (Table 1).

Amoxicillin can be synthesized using D-HPGA as the acyl donor and 6-APA as the acyl acceptor. Strikingly, PA from *P. rettgeri* turned out to be the most active wild-type enzyme in this reaction, whereas it displayed the lowest synthetic activity in the synthesis of ampicillin when the amide of phenylacetic acid was used as acyl donor. The three hybrids had the highest values for the initial synthesis/hydrolysis ration and the highest level of product accumulation.

Table 3

Steady state kinetic parameters for hydrolysis of ampicillin and D-phenylglycine amide (D-PGA)

Penicillin acylase	Ampicillin			D-PGA		
	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ (mM <sup>-1</sup> s <sup>-1</sup> )	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ (mM <sup>-1</sup> s <sup>-1</sup> )
<i>E. coli</i>	36.7	3.6	10.19	35.6	27.4	1.30
<i>K. cryocrescens</i>	17.2	3.0	5.83	41.7	90.5	0.46
<i>P. rettgeri</i>	20.6	5.8	3.55	39.7	64.9	0.61
6G8	29.6	2.7	10.96	42.6	43.2	0.99
73C4	22.1	3.9	5.67	28.2	57.5	0.49
6B11	25.7	6.3	4.08	28.9	45.3	0.64

Reaction conditions are described in Section 2.

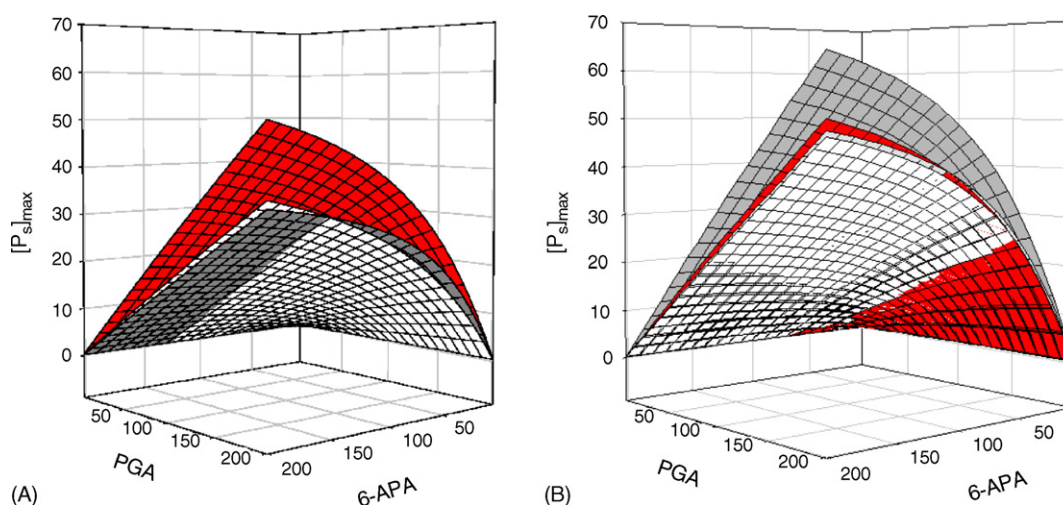


Fig. 5. Calculations of the maximum level of ampicillin accumulation ( $[P_s]_{\max}$ ) of the three parent enzymes (A) and the three selected hybrids and *E. coli* PA (B), using Eq. (3). (A), the simulated  $[P_s]_{\max}$  of *E. coli* PA is shown in red, *K. cryocrescens* PA in grey and *P. rettgeri* PA in white. (B), the simulated  $[P_s]_{\max}$  of *E. coli* PA is shown in red, 6G8 in light grey, 73C4 in white and 6B11 in dark grey.

Cephalexin synthesis was tested using D-PGA as the acyl donor and 7-ADCA as the acyl acceptor (Table 1) and it appeared that this conversion is catalyzed more efficiently than the synthesis of ampicillin as  $[P_s]_{\max}$  and  $(v_p/v_{p_h})_{\text{ini}}$  were considerably higher, which is in agreement with previous observations [16,26]. The  $(v_p/v_{p_h})_{\text{ini}}$  of *K. cryocrescens* PA was higher than that of *E. coli* and *P. rettgeri* PAs, but the three hybrids showed the best values.

For the synthesis of cefadroxil, D-HPGA was used as the acyl donor and 7-ADCA as the nucleophile (Table 1). In this reaction, the three hybrids showed increased  $(v_p/v_{p_h})_{\text{ini}}$  and  $[P_s]_{\max}$  values compared to the parent enzymes, of which the PA from *P. rettgeri* appeared to be the most active. This is in agreement with the results obtained with the synthesis of amoxicillin, in which D-HPGA was also used as acyl donor, indicating that PA of *P. rettgeri* performs better with D-HPGA than with D-PGA as acyl donor. The synthetic activity of hybrid 6G8 was higher in all synthesis reactions than that of PA from *E. coli*, whereas the synthetic activity of the other hybrids was between 62 and 108%.

### 3.4. Steady-state kinetic parameters

To investigate the hybrids in more detail, steady-state kinetic parameters for the chromogenic substrates NIPAB and NIPGB were determined. The data in Table 4 show that the kinetic

parameters of the hybrids for NIPAB had not changed much in comparison with those of PA from *E. coli*, indicating that the positional changes of the active site residues are relatively small. The  $K_m$  of hybrid 6G8 was decreased as compared to *E. coli* PA, resulting in an increased specificity for NIPGB, whereas hybrid 6B11 showed a 50% increase of the  $K_m$ .

Since 6-APA used for synthesis of ampicillin and amoxicillin may contain traces of phenylacetic acid (PAA) and the  $K_i$  of PA for PAA is in the micromolar range, the inhibition constant for PAA is an important parameter. It appeared to be two-fold lowered for hybrid 6G8 as compared to *E. coli* PA, making this hybrid enzyme more sensitive to inhibition by PAA. However, the other two hybrids did not show this reduction. Hybrid 6B11 even displayed a small increase of the  $K_i$  for PAA, making it less prone to PAA inhibition. The combination of this characteristic and the improved synthetic properties make hybrid 6B11 a good candidate for the production of ampicillin.

### 3.5. DNA and amino acid sequence of the improved mutants

DNA sequencing showed that the recombinants were hybrids of the *E. coli* and *K. cryocrescens* PA encoding genes (Fig. 6). The length of the overlapping DNA regions where crossing over had occurred varied from 2 to 30 bp with an average size of 15 bp.

Table 4  
Steady-state kinetic parameters for hydrolysis of NIPAB and NIPGB and the inhibition constant for PAA

Penicillin acylase	NIPAB			NIPGB			PAA
	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1} \text{s}^{-1}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1} \text{s}^{-1}$ )	$k_i$ ( $\mu\text{M}$ )
<i>E. coli</i>	22.7	16.2	1.40	16.5	1000	16.5	28.2
6G8	16.5	11.3	1.46	18.0	640	28.1	16.0
73C4	18.4	16.7	1.10	12.7	1040	12.2	28.5
6B11	21.9	18.4	1.19	18.3	1490	12.3	30.3

Reaction conditions are described in Section 2.



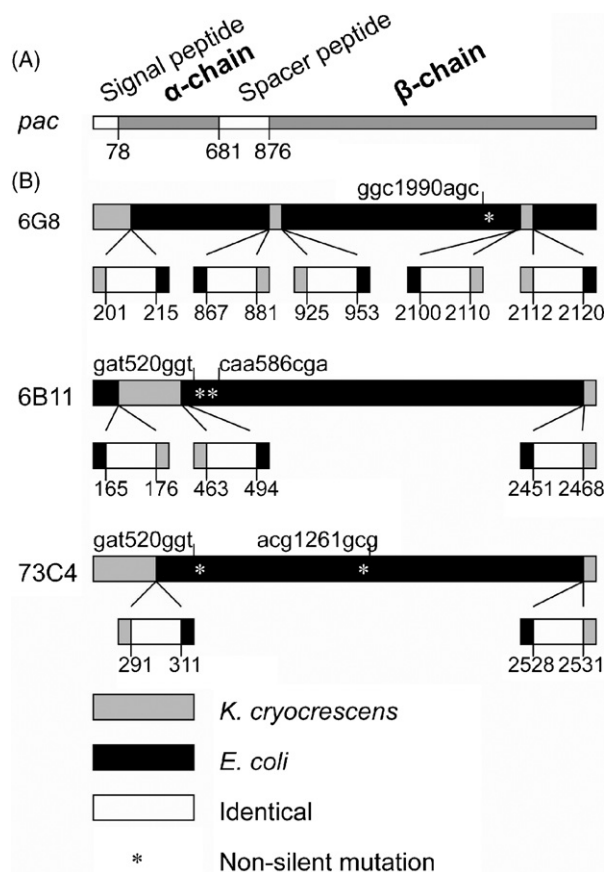


Fig. 6. Schematic representation of the *pac* gene of *E. coli* PA (A) and hybrid penicillin acylases (B). (A) The *E. coli* *pac* gene. The numbers indicate the base pair (bp) of the gene encoding the preprotein. (B) Segments originating from *E. coli* *pac* are shown in black, those from *K. cryocrescens* in grey, and identical parts in white. DNA segments where crossing over occurred are enlarged and the bp numbers are given. The asterisks indicate non-silent mutations and the codons of these mutations are given.

The *E. coli* gene coding for PA consists of 2541 bp, whereas the gene of *K. cryocrescens* is 6 bp shorter. The three chimeric genes were composed of 2541 bp and a greater part of the genes originates from *E. coli*. The signal peptide of hybrids 6G8 and 73C4 originates from *K. cryocrescens*. Moreover, one of the segments of *K. cryocrescens* in hybrid 6G8 encoded the N-terminal active site serine. In addition to DNA crossovers, point mutations were introduced during the amplification and reassembly, with an overall frequency of 0.20% in the three hybrids (Fig. 6). Notably, one mutation coding for mutation  $\alpha$ D148G was found in the genes of 6B11 and 73C4. In both sequences the same codon was found at position 148, which could indicate that this mutation was introduced in the initial amplification of the parent genes. Other mutations that were present in the hybrid enzymes were residues  $\beta$ G375,  $\alpha$ Q170 and  $\beta$ T132. These residues are located far from the substrate binding site in enzyme regions not known to be involved in catalysis.

#### 4. Discussion

Different ways have been explored to improve the biocatalytic production of semi-synthetic  $\beta$ -lactam antibiotics by penicillin

acylase, such as optimising the pH of the reaction medium [27], the use of immobilized PA [28,29], addition of cosolvents [30], and the use of high concentrations of the  $\beta$ -lactam nucleus [31,32]. Less work has been devoted to the improvement of the biocatalyst itself [16,33,34]. In this paper, we have explored the use of family gene shuffling [22,23] of the PA encoding genes from *E. coli*, *K. cryocrescens* and *P. rettgeri* to obtain improved enzyme variants that do not show the undesirable reduction of catalytic efficiency found with earlier mutants of *E. coli* PA.

Of the three enzymes that were used, PA from *E. coli* proved to be the best enzyme for the synthesis of ampicillin, which is in accordance with the literature [26,35]. PA from *P. rettgeri* appeared to be a poor enzyme for the synthesis of both ampicillin and cephalexin with D-PGA as the acylating agent, but it was the best enzyme for the synthesis of amoxicillin and cefadroxil with D-HPGA as acyl donor. The *E. coli* and *K. cryocrescens* PAs were also found to be more suitable for the production of cephalexin than the *A. faecalis* or *P. rettgeri* enzymes in a recent study by Cheng et al. [36].

Sequence comparison of the three enzymes shows that the hydrophobic pocket of *P. rettgeri* PA that binds the acyl moiety of the substrate, including the hydroxyl group of D-HPGA, differs only in one amino acid from that in the other two enzymes. This residue,  $\beta$ 56, is a leucine in PA from *P. rettgeri*, whereas it is a valine in the PAs from *E. coli* and *K. cryocrescens*. Atom CG1 of  $\beta$ V56 is 3.90 Å away from the oxygen atom of the hydroxyl group of *p*-hydroxyphenylacetic acid in structure 1AI6.PDB of *E. coli* PA [37], indicating that the presence of a small residue at this position may create space for the hydroxyl group of *p*-hydroxyphenylacetic acid and D-HPGA. Recently, it was shown that mutation  $\alpha$ M142L, at a position that is located next to residue  $\beta$ L56 in the substrate binding site, increased the selectivity for 6-bromohexanamide in PA of *P. rettgeri* by creating more space for the sterically hindered bromine aliphatic derivatives [46].

After gene shuffling, we used plate screening and selection to obtain active enzyme. The percentage of active transformants obtained (3%), as judged by liberation of L-leucine in the growth assay, was lower than the 20% reported for gene shuffling in other papers [38,39]. However, Zhou et al. [35] found that 5% of their library encoded active enzymes, whereas gene shuffling of expandase yielded a library of which most clones had low or no detectable activity [40]. Sizmann et al. [41] showed that mutations at the C-terminus of *E. coli* PA as well as internal deletions within the  $\beta$ -subunit prevented correct folding of the mature protein. Furthermore, the yield of folded protein was significantly reduced when mutations were introduced in the spacer peptide, indicating that PA could be relatively sensitive to mutations because production of active enzyme may be corrupted at different stages and loss of activity can be due to mutations in different parts of the enzyme.

Further screening of the active PAs by HPLC revealed that 94% of the 700 active transformants converted D-PGA and 6-APA to ampicillin. However, only 3% of these transformants appeared to be improved in the rate of synthesis. This percentage seems low, but previous work has shown that most mutants of *E. coli* PA and mutants of a PA obtained from a environmen-

tal gene library that had enhanced values for  $(v_p/v_h)_{ini}$  and  $[P_s]_{max}$  have a greatly reduced synthetic rate [16,33,42]. Since we only selected variants with a high synthetic activity, such less-active mutants were discarded during the selection. The axiom “you get what you screen for” is often regarded as the first law in directed evolution [43,44]. Notwithstanding the axiom, the three best hybrids which were selected on basis of enhanced ampicillin synthesis were also improved in the production of amoxicillin, cephalixin and cefadroxil. This might be explained by the resemblance between these  $\beta$ -lactam antibiotics. Obviously, screening for such other products may well yield hybrids with even better synthetic properties than the ones we report here.

Sequence analysis of the genes that encode the best synthetic enzymes, 73C4, 6B11 and 6G8, showed that they were hybrids of *E. coli* and *K. cryocrescens* PA genes (Fig. 6). The three hybrid genes did not contain parts of the PA-encoding gene from *P. rettgeri*, which showed the least favourable values for the synthesis of ampicillin. In addition, random mutations were found in the hybrid genes, most of which are located far from the active site. It is expected that positional changes of the residues in the active site due to these mutations are small. They might, however, influence the accessibility of the substrate binding site. The carbonyl oxygen atom of residue  $\beta$ G375 is located 13.5 Å from both carboxylic oxygen atoms of penicillin G in the crystal structure of 1FXV.PDB and is positioned in the entrance to the substrate binding site. Residue  $\beta$ G375 was mutated in hybrid 6G8 to a serine. To investigate the contribution of this mutation to the properties of hybrid 6G8 in ampicillin synthesis from 6-APA and phenylglycine amide, we have constructed mutant  $\beta$ G375S and it displayed an increase of the  $[P_s]_{max}$  of 22%, whilst the  $(v_p/v_h)_{ini}$  was elevated by 80%. Moreover, this mutant did not show any decrease of the synthetic activity (details not shown). These findings are remarkable in view of the distance between this residue and the active site. Similarly, we have constructed mutant  $\alpha$ D148G, carrying a mutation that was found in two different improved hybrids. The  $[P_s]_{max}$  of this mutant in ampicillin synthesis increased 1.2-fold and the  $(v_p/v_h)_{ini}$  was increased by 80% as compared to *E. coli* wild-type PA, indicating that residue  $\alpha$ D148 is a good target for site-directed mutagenesis (details not shown). This amino acid is located next to residues  $\alpha$ R145 and  $\alpha$ F146, which undergo a conformational change upon substrate binding [33,37,45]. Residue  $\alpha$ D148 is hydrogen bonded to three conserved residues, possibly giving a tight connection and replacing it may increase the flexibility of the part of the enzyme that is involved in the induced-fit repositioning.

Of the three enzymes that were included in the gene shuffling, PA from *E. coli* displayed slightly higher  $[P_s]_{max}$  and  $(v_p/v_h)_{ini}$  values for the synthesis of ampicillin than PA of *K. cryocrescens*, whereas the latter enzyme displayed a higher synthetic rate. Interestingly, hybrids of these two enzymes showed increased values for all three parameters ( $[P_s]_{max}$ ,  $(v_p/v_h)_{ini}$  and  $v_p$ ), as compared to both parent enzymes, and the best hybrids were calculated to show improved ampicillin production over a range of concentrations of the  $\beta$ -lactam. Thus, gene shuffling was an useful method for improving the synthetic properties of penicillin

acylase. Moreover, gene shuffling yielded substitutions that contributed to improved synthesis, underlining that it may enhance biocatalyst performance not only by recombination but also by introducing point mutations, which in turn leads to identification of new targets for site-directed mutagenesis, which might not have been found by scrutinizing the crystal structure of the enzyme.

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